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## ORIGINAL ARTICLE

# Impact of hydroquinone used as a redox effector model on potential denitrification, microbial activity and redox condition of a cultivable soil



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## KEYWORDS

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Hydroquinone doses

**Abstract** In this microcosm study, we analyzed the effect produced by hydroquinone on the expression of soil biological denitrification, in relation to the redox state of the soil, both in terms of intensity factor ( $Eh'$ ) and capacity factor (amount of oxidized or reduced compounds).

The supplementation of an Argiudoll soil with hydroquinone decreased the soil apparent reduction potential ( $Eh'$ ) and soil dehydrogenase activity (formazan production from tetrazolium chloride reduction; redox capacity factor), the relationship between both factors being highly significative,  $r=0.99$  ( $p<0.001$ ). The bacterial population (measured by colony forming units) increased, and the production of  $N_2O$  was greater ( $p<0.001$ ) at 200 and 400  $\mu\text{g/g}$  dry soil doses. Furthermore, there was an inverse relationship between soil dehydrogenase activity and the number of bacteria ( $r=-0.82$ ;  $p<0.05$ ), increased denitrification activity and changes in the  $\text{CO}_2/\text{N}_2\text{O}$  ratio value. These results suggest that hydroquinone at supplemented doses modified the soil redox state and the functional structure of the microbial population. Acetate supplementation on soil with hydroquinone, to ensure the availability of an energy source for microbial development, confirmed the tendency of the results obtained with the supplementation of hydroquinone alone. The differences observed at increased doses of hydroquinone might be explained by differences on the hydroquinone redox species between treatments.

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**PALABRAS CLAVE**

Óxido nítrico;  
Condiciones redox;  
Actividad  
deshidrogenasa;  
Microorganismos  
heterótrofos;  
Dosis de hidroquinona

## El impacto de la hidroquinona usada como un modelo de efector redox sobre la desnitrificación potencial, la actividad microbiana y las condiciones redox de un suelo cultivable

**Resumen** En este trabajo estudiamos, en condiciones de microcosmos, el efecto que produce la hidroquinona sobre la expresión de la desnitrificación en relación con el estado de óxido-reducción del suelo, en términos de factor de intensidad ( $E_h'$ ) y de factor de capacidad (cantidad de compuestos oxidados o reducidos).

La suplementación de un suelo argiudol con hidroquinona disminuyó el potencial de reducción aparente ( $E_h'$ ) y la actividad deshidrogenasa (producción de formazán a partir de la reducción de cloruro de tetrazolio; factor de capacidad redox), la relación entre ambos factores fue altamente significativa,  $r = 0,99$  ( $p < 0,001$ ). La población bacteriana heterotrófica (medida como unidades formadoras de colonias) aumentó y la producción de  $N_2O$  fue mayor ( $p < 0,001$ ) con las dosis de 200 y 400  $\mu\text{g/g}$  de suelo seco. Además se observó una relación inversa entre la producción de formazán y el número de bacterias ( $r = -0,82$ ;  $p < 0,05$ ), la actividad desnitrificadora aumentó y se produjeron cambios en el valor del cociente  $\text{CO}_2/\text{N}_2\text{O}$ . Estos resultados sugieren que la hidroquinona, en las dosis empleadas, modificó el estado redox del suelo y la estructura funcional de la población microbiana. La suplementación con acetato en el suelo con hidroquinona, a fin de asegurar la disponibilidad de una fuente de energía para el desarrollo bacteriano, confirmó la tendencia de los resultados obtenidos con la suplementación con hidroquinona solamente. Las diferencias observadas con el incremento en la dosis de hidroquinona podrían explicarse por las diferencias sobre las especies redox de la hidroquinona entre los tratamientos.

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## Introduction

Soil phenolic compounds and their derivatives are originated in the soil by the degradation of microorganisms, plants and other soil humic substances. In agro-ecosystems they may be added through agrochemicals or their degradation products. Hydroquinone (HQ) is one of the most important phenolic compounds in soil. It is a quinone type found mainly as a lignin constituent<sup>25</sup> and together with semiquinones, they are the main redox reactive groups of humic substances in soil.<sup>8</sup> Their oxidation, even within the humic substance group, produces semiquinone and quinone, creating a redox cycle, with electron and proton transfer among different electroactive species ( $\text{QH}_2 \rightleftharpoons \text{QH} \rightleftharpoons \text{Q}$ ), minerals and microbial components in soil.<sup>16,29</sup> This speciation behavior is conducted by several physicochemical conditions of the system, among them acid-base state and redox state,<sup>20</sup> and affect the amount of redox effectors (redox capacity factor) and the apparent redox potential value ( $E_h'$ , redox intensity factor). These redox species may act differently upon biological and non-biological processes that involve e-transfers, i.e., denitrification or  $\text{Fe}^{2+}$  oxidation.<sup>22,28</sup>

In certain environments such as water, HQ has a direct toxic effect on microorganisms.<sup>4,6</sup> In complex microbial environments, such as in the humic-mineral matrix of soil, HQ interacts with minerals and there is not such a direct effect on microorganisms. HQ is known to be degraded through microbial activity,<sup>12,31</sup> to undergo oxidation by light<sup>37</sup> or minerals when it is in contact with soil clay-humic structures, and to get involved in humic substance polymerization.<sup>13</sup>

Denitrification is a process mediated by a great variety of microorganisms which respire nitrate (use  $\text{NO}_3^-$  as electron final acceptor), produce  $\text{NO}_2^-$  and N oxides among them  $\text{N}_2\text{O}$ .<sup>11</sup> The relevance of  $\text{N}_2\text{O}$  deserves great attention due to the fact that  $\text{N}_2\text{O}$  shows high reactivity over the ozone layer. According to estimations made by Revell and his collaborators,<sup>26</sup> in the coming years,  $\text{N}_2\text{O}$  may be one of the gases having the greatest negative influence on the greenhouse effect. Moreover, the denitrification process is relevant for its negative effect on soil fertility due to the extraction of nitrogen from the soil.<sup>11</sup>

In soil, microbial respiration generates many redox reactions that transfer  $e^-$  to  $\text{NO}_3^-$ -R,<sup>35</sup> free radicals and  $\text{H}_2\text{O}_2$ ,<sup>10</sup> or to acceptors introduced as triphenyl tetrazolium chloride (TTC), which is reduced to triphenyl formazan (TPF).<sup>9</sup> The TPF production was also used to determine the number of heterotrophic microorganisms<sup>2</sup> from the microbial dehydrogenase activity since TTC may act as substrate for these enzymes.<sup>32,36</sup> In other words, in our study, the same reaction (TTC reduction) may inform on two related processes (i) changes in the redox capacity of the system, and (ii) quantify the evolution of the heterotrophic microbial population.

In this research, we studied the pathways through which HQ affects denitrification expression, in relation to soil oxidation-reduction processes in terms of both redox intensity ( $E_h'$ ) and redox capacity (amount of TPF produced, number of oxidized or reduced compounds). The study was carried out under controlled laboratory conditions, and the parameters assessed were the number of heterotrophic bacteria, dehydrogenase activity, apparent reduction

potential ( $E_h'$ ), and potential  $N_2O$  and  $CO_2$  production in soil. Acetate as carbonated substrate was supplemented.

## Materials and methods

### Soil treatment

The loamy-clay soil used in this study is a Typic Argiudoll (Peyrano series) from naturalized grassland located in Casilda (province of Santa Fe, Argentina). Soil was sieved through an ASTM N 5 (4000  $\mu m$ ) and taken from the surface layer (0–15 cm) after removing the top surface layer; the natural plant remains were eliminated by hand. The soil was stored at  $+4^\circ C$  for a maximum of 1 week with field-moist soil (25%). The main properties of the soil included 37 g/kg organic matter, 2.3 g/kg total-N, pH 6.1, and 27 cmol<sub>c</sub>/kg cation exchange capacity.

### Experimental procedure

Soil (10 g oven-dry basis) was introduced into 22 ml-tubes. Humidity was adjusted to 100% (p/p) using sterilized deionized water and HQ solution stock and acetate solution, depending on the experiment.

*Experiment 1* was conducted to study the effects of HQ supplementation alone. The HQ (Sigma-Aldrich®, CAS number 123-31-9) stock solution was added to obtain final concentrations of 0 (control), 100, 200 and 400 microgram per gram of dry soil ( $\mu g/g$ ).

*Experiment 2* was conducted to study the effects of HQ supplementation in the presence of a source of carbon and electrons, which was done by adding acetate solution (Sigma-Aldrich®, CAS number 127-09-3) 1 milligram per gram of dry soil in each tube with soil. Afterwards, a HQ stock solution was added to obtain final concentrations of 0 (control), 100, 200 and 400  $\mu g/g$ .

Tubes were capped with Suba Seal stoppers. All of the experimental treatments were prepared in triplicate and the tubes were placed in a dark chamber at  $25^\circ C$  for 6 days. At this time samples of each treatment were collected and employed to assess the biological tests and physicochemical analysis.

### Biological tests

**Soil dehydrogenase activity:** Dehydrogenase activity was determined by the reduction of TTC according to the procedure proposed by Casida *et al.*<sup>2</sup> Samples of 1 g were placed into a 18 ml-tube; then 0.01 g  $CaCO_3$  and 1 ml of 2,3,5-triphenyl tetrazolium chloride (Sigma-Aldrich®, CAS number 298-96-4) in a water solution (3%) were added. Tubes were incubated at  $30^\circ C$  in a dark chamber. After 20 h, acetone was added and the soil suspension was agitated and filtered. The red solution was measured at 485 nm and compared with the TPF standard.

**Total heterotrophic bacteria count:** Samples (1 g) were collected from each treatment and serial dilutions of each subsample were spread on Luria Broth (LB) agar plates, according to the procedure in Fracchia *et al.*<sup>7</sup> The plates were incubated for 48 h at  $30^\circ C$  in a dark chamber. Total

heterotrophic bacteria were counted and expressed as colony forming units (CFUs) per gram of dry soil.

**Denitrification activity:** Samples (1 g) of each treatment were placed in a 10 ml-tube capped with septa for gas extraction under anaerobic conditions, and 1 ml sterilized water containing  $KNO_3$  (1 mg N/g dry soil) was added. The atmosphere of each tube (2'-vacuum) was replaced by  $N_2$  to provide anaerobic conditions.  $N_2$  (10%) was replaced by  $C_2H_2$  to inhibit  $N_2O$  reductase activity of the soil denitrifying microorganisms.<sup>38</sup> Tubes were placed in a dark chamber at  $30^\circ C$  during 20 h. The headspace atmosphere was sampled to quantify  $N_2O$  and  $CO_2$  production. Gases were analyzed on a gas chromatograph equipped with a thermal conductivity detector (Konik Instruments S.A., Barcelona, Spain) and a stainless steel column packed with Porapak Q and  $N_2$  as carrier gas.

### Physicochemical analysis

**Apparent redox potential ( $E_h'$ )**<sup>20</sup> was measured with a platinum combined electrode (Orion 96-02). The electrode measurements were calibrated using a solution containing 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, and 100 mM KCl.<sup>39</sup> The 242 mV reference electromotive force was added to the actual electromotive force.<sup>21</sup>

### Data treatment

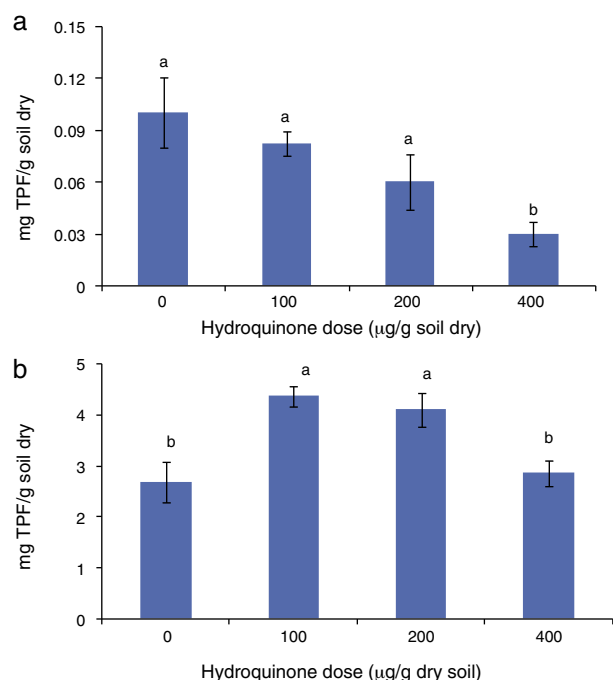
All results are expressed in terms of weight oven-dry soil. The data were statistically analyzed with the variance analysis; when a significant *F*-value was detected, the Tukey Test was used to separate the treatment means.<sup>23</sup> The Pearson correlation coefficient was used to analyze the relationship between biological and physicochemical parameters and HQ doses, and between dehydrogenase activity and CFUs.

## Results and discussion

### Soil dehydrogenase activity and number of colony forming units

The soil dehydrogenase activity assessed by TTC reduction decreased when the HQ dose was increased (Fig. 1a and b). This was observed in both experiments, i.e., in soil with HQ alone and soil with HQ plus acetate. The highest dose of HQ decreased the TTC reduction ( $p < 0.05$ ). The soil supplemented with acetate showed, in general, a higher dehydrogenase activity with respect to the soil without acetate. The relationship between soil dehydrogenase activity and HQ doses plus acetate also showed a negative and statistically significant coefficient correlation,  $r = -0.99$  and  $r = -0.98$  ( $p < 0.001$ ) in soil with HQ alone and in soil with HQ plus acetate, respectively.

An assessment of HQ supplementation on a soil heterotrophic bacterial population was carried out by counting the CFUs and measuring soil dehydrogenase activity. Although some authors describe HQ as a toxic compound for microorganisms such as *Bacillus subtilis* in aquatic ecosystems,<sup>4</sup> and also for microorganisms in a Chinese fir forest soil,<sup>3</sup> the results show that HQ did not reduce CFUs

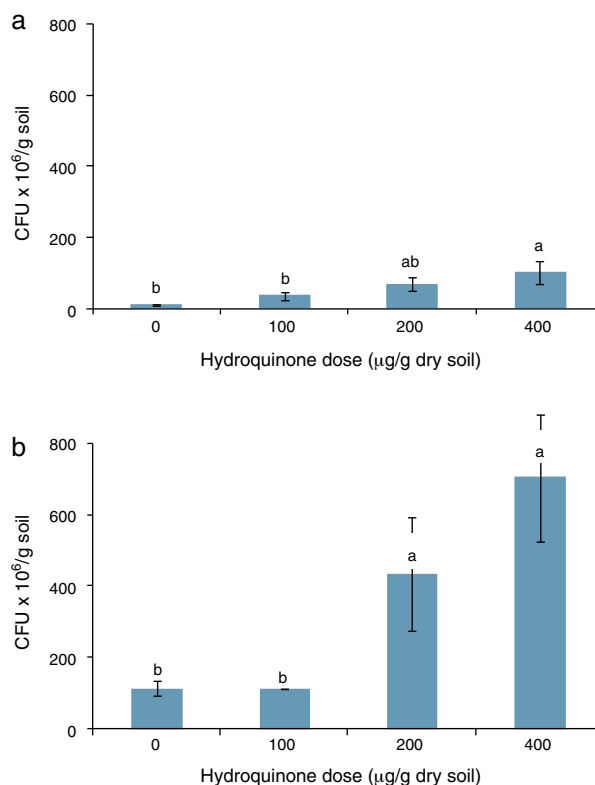


**Figure 1** Soil dehydrogenase activity as triphenyl formazan (TPF) production in (a) soil supplemented with hydroquinone alone and (b) soil supplemented with hydroquinone plus acetate (1 mg/g dry soil). Bars labeled with the same letter were not significantly different from each other ( $p < 0.05$ , Tukey's test).

in soil; on the contrary, CFUs increased with HQ supplementation. The units increased from  $3.60 \times 10^7$  to  $1.02 \times 10^8$ /g dry soil when 100, 200 and 400 µg HQ per gram of dry soil were added (Fig. 2a).

Moreover, acetate supplementation increased the number of CFUs, which was between  $1.1 \times 10^8$  and  $7.4 \times 10^8$ /g dry soil, when 100, 200 and 400 µg per gram of dry soil HQ doses and acetate were added (Fig. 2b). There was a correlation between HQ doses and CFU increase in both experiments,  $r = 0.81$  ( $p < 0.05$ ) and  $r = 0.84$  ( $p < 0.05$ ) in experiments 1 and 2, respectively. The number of CFUs was statistically greater ( $p < 0.05$ ) with higher doses of HQ alone (Fig. 1a) and also with HQ and acetate (Fig. 1b). In complex microbial environments, such as the humic-mineral matrix of soil, HQ may be degraded<sup>12</sup> or it may interact with minerals involved in humic substance polymerization,<sup>13</sup> and consequently, it does not have a direct negative effect on soil heterotrophic microorganisms.

Soil dehydrogenase activity was described and employed by many authors as an indicator of soil microbial activity.<sup>1,9,18,19,34</sup> In this study, the relationship between CFUs and soil dehydrogenase activity was inverse,  $r = -0.82$  ( $p < 0.05$ ) in soil supplemented with HQ alone and  $r = -0.79$  ( $p < 0.05$ ) in soil supplemented with HQ plus acetate. These results clearly indicate that in the studied systems, the dehydrogenase activity did not express the activity of the heterotrophic bacterial colony assessed. This result agrees with reports from different authors which indicate, in some cases, that TPF underestimates the actual dehydrogenase activity.<sup>17</sup> On the other hand, authors such as Praveen-Kumar and Tarafdar<sup>24</sup> showed that not every



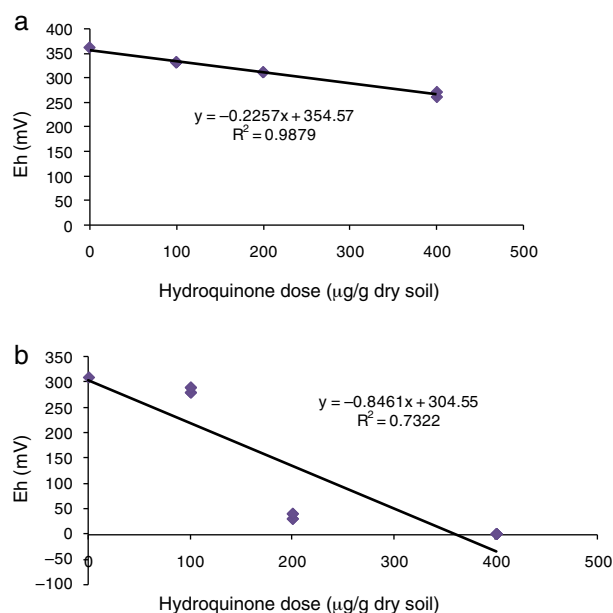
**Figure 2** Count of soil heterotrophic bacteria (CFUs) in (a) soil supplemented with hydroquinone alone, and (b) soil supplemented with hydroquinone plus acetate (1 mg/g dry soil). Bars labeled with the same letter were not significantly different from each other ( $p < 0.05$ , Tukey's test).

microorganism colony in soil can reduce TTC. The results obtained in our research are significant because they may show that quinone compounds, which are abundant in soil, are potential effectors in the relationship between TTC reduction and the number of bacteria in soil.

### Study of soil redox state

Soil redox intensity, measured by apparent oxidation-reduction potential (Eh'), decreased at the highest HQ dose (400 µg) (Fig. 3). Eh' values in soil supplemented with HQ alone were microaerophilic conditions,<sup>16</sup> between 330 mV at the lowest dose and 265 mV at the highest dose (Fig. 3a). In soil supplemented with acetate, there were greater reduction conditions, between 270 and 0 mV (Fig. 3b). The relationship between these values and HQ concentration provided a negative correlation coefficient,  $r = -0.99$  ( $p < 0.001$ ) in soil supplemented with HQ alone, and  $r = -0.82$  ( $p < 0.05$ ) in soil supplemented with HQ plus acetate.

In addition, the slopes of linear functions (Fig. 3a and b) provided significant data in both experiments, i.e., the figures given by the slope in soil supplemented with HQ alone was  $-0.22$ , whereas in soil supplemented with HQ plus acetate it was  $-0.84$ . Therefore, the reduction potential was four times lower in soil supplemented with HQ plus acetate than in the treatments of soil supplemented with



**Figure 3** Relationship between apparent redox potential (Eh') and hydroquinone doses in (a) soil supplemented with hydroquinone alone, and (b) soil supplemented with hydroquinone plus acetate (1 mg/g dry soil).

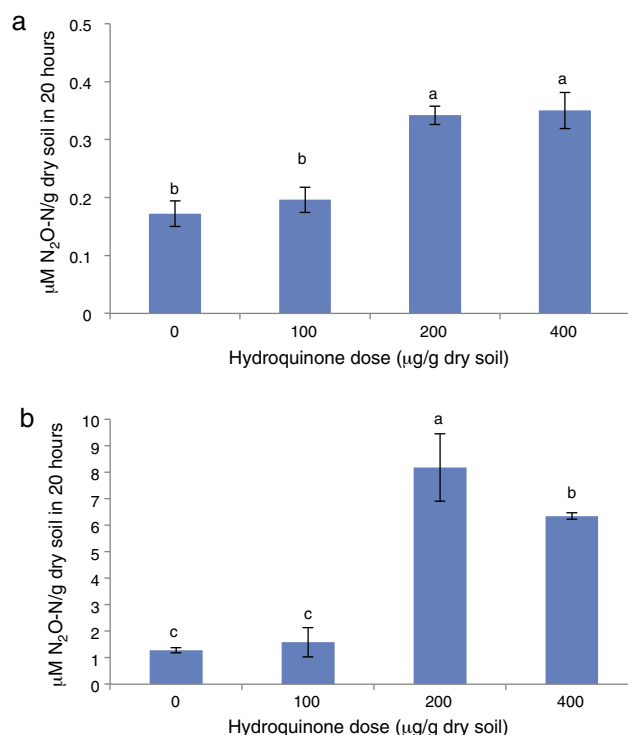
HQ alone. This shows that the availability of a carbon source such as acetate – which is capable of being easily assimilated – greatly increased soil reduction conditions.

Microbial reduction ability, defined by TPF production as described above (Fig. 1a and b), tended to decrease with the addition of higher HQ doses. Some authors such as Schmidt and Burnett<sup>30</sup> described microbial cell reduction ability as the amount of TPF produced in a bacterial culture. In this study, dehydrogenase activity – the amount of formazan produced – becomes a descriptor of soil-redox-capacity-state.

There was a very close relationship between redox intensity (Eh') and microbial reduction ability (TPF production),  $r = 0.99$  ( $p < 0.001$ ) and  $r = 0.72$  ( $p < 0.05$ ) in soil only supplemented with HQ and in those with HQ plus acetate, respectively. This intracellular redox activity requires electron-proton availability to reduce TTC. Therefore, the lower soil dehydrogenase activity observed in the treatments at the highest HQ doses might be due to the fact that (i) the reducing metabolism of the cell might have reduced other compounds<sup>27,36</sup> such as quinones or semiquinones, and (ii) in soil supplemented with HQ alone and in soils supplemented with HQ plus acetate a microaerophilic population with respiratory chains that do not use  $O_2$  as a terminal electron acceptor might have developed, so that the reduction-power produced was lower than the reducing power of TTC, and consequently, TTC reduction was also low.

### Effect on potential denitrification

Soil  $N_2O$  production caused by  $NO_3^-$  reduction results mainly from microbial denitrification activity.  $N_2O$  production was measured by introducing an amount of  $KNO_3$  which would not limit the activity expression, so as to quantify the potential



**Figure 4** Soil  $N_2O$  production in (a) soil supplemented with hydroquinone alone and (b) soil supplemented with hydroquinone plus acetate (1 mg/g dry soil). Bars labeled with the same letter were not significantly different from each other ( $p < 0.05$ , Tuckey's test).

expression of denitrifying microorganisms under potential conditions<sup>14</sup> (see Materials and methods section).  $NO_3^-$  acts as a terminal electron acceptor in denitrifying microorganisms in the absence of  $O_2$  and in the presence of acetylene the final product is  $N_2O$ .<sup>33</sup>

As may be observed in Fig. 4a,  $N_2O$  production increased when the HQ doses were higher ( $p < 0.05$ ) and the acetate supplementation increased the  $N_2O$  production as observed by Lescure *et al.*<sup>15</sup> When comparing  $N_2O$  production values in treatments without and with acetate supplementation (Fig. 4a and b, respectively), the addition of this carbonated source increased  $N_2O$  production approximately 80–95 % in accordance with the HQ dose added.

In addition, in systems supplemented with HQ plus acetate, the treatment with 200  $\mu g/g$  showed the greatest  $N_2O$  production, which was up to four times greater than the control without HQ (Fig. 4a), and the treatment at the highest HQ dose (400  $\mu g/g$ ) showed a lower  $N_2O$  production, although greater than the control and the treatment with 100  $\mu g/g$  (Fig. 4b).

Carbon dioxide production in soil supplemented with 200  $\mu g/g$  HQ plus acetate was slightly greater than in the other treatments ( $p > 0.05$ ) (Table 1). When  $CO_2$  production was compared in the experimental soils, with and without acetate, this compound seems to have increased  $CO_2$  production, which was four times greater than in the non-supplemented experimental soil. This increase in C-reduction activity may be caused by the higher number of heterotrophic bacteria (CFUs) observed (Fig. 2a and b).



**Table 1** CO<sub>2</sub> production and CO<sub>2</sub>/N<sub>2</sub>O ratio in soil supplemented with hydroquinone

Hydroquinone <sup>a</sup>	CO <sub>2</sub> <sup>b</sup>		CO <sub>2</sub> /N <sub>2</sub> O ratio	
	Experiment 1 <sup>c</sup>	Experiment 2 <sup>d</sup>	Experiment 1	Experiment 2
0	1.59 a ± 0.09	6.12 a ± 0.38	9.35 a	4.78 a
100	1.64 a ± 0.10	7.05 a ± 0.15	8.63 a	4.49 a
200	2.02 a ± 0.23	6.5 a ± 0.9	5.17 b	0.79 b
400	1.65 a ± 0.009	4.71 b ± 0.6	4.5 b	0.74 b

<sup>a</sup> Hydroquinone dose (μg/g dry soil).<sup>b</sup> Soil CO<sub>2</sub> production (μmol/g dry soil).<sup>c</sup> Experiment 1: soil supplemented with hydroquinone alone.<sup>d</sup> Experiment 2: soil supplemented with hydroquinone plus acetate (1 μg/g dry soil). Values are means of three replications. Significant differences between treatments (within a column) at  $p=0.05$  (Tukey's test) are indicated by different letters.

Table 1 shows CO<sub>2</sub>/N<sub>2</sub>O ratios obtained from samples of Experiments 1 and 2. The ratio represents the stoichiometric relationship between N<sub>2</sub>O and CO<sub>2</sub> production in a denitrification process, with a theoretical value that corresponds to each electron-donor compound. In this study, the main supplemented electron source was acetate and the theoretical value of this stoichiometric ratio is two.<sup>5</sup> If the values differ from this theoretical value, the ratio shows that there are differences in intensity and/or diversity in metabolic pathways that may produce these gases, including the biological denitrification process.<sup>21</sup> Table 1 shows that in both experiments, the ratio was lower when the HQ dose was higher, and that an acetate supplementation lowered the ratio value in relation to the treatments without acetate. This suggests that a greater NO<sub>3</sub><sup>-</sup> reduction (Fig. 4a and b) will not depend directly on a higher electron availability provided by an exogenous carbon source. On the other hand, as CO<sub>2</sub> values were relatively constant in the treatments with and without acetate, the systems may be showing that electron sources behave differently for NO<sub>3</sub><sup>-</sup> reduction. Those compounds might have come from fermentation routes stimulated by the presence of HQ or from self-oxidation of supplemented HQ, whose oxidized end product might not be CO<sub>2</sub>. This may explain why CO<sub>2</sub> production was relatively constant and N<sub>2</sub>O production was greater when the HQ concentration was increased.

The supplementation of HQ in the studied soil affected the reduction conditions of the system in relation to redox intensity (Eh') and reduction capacity (TPF production), and with the HQ supplementation plus acetate in soil these results were confirmed. An inverse relationship between TPF production and the number of microorganisms (CFUs), as well as changes in the CO<sub>2</sub>/N<sub>2</sub>O ratio value may confirm that the HQ supplementation modified the functional structure of the microbial population. In addition, HQ supplementation favored the development of a heterotrophic population (shown by CFUs) and the expression of a microbial population, such as the denitrifying population, which does not use O<sub>2</sub> as terminal electron acceptor.

Finally, as the supplementation of acetate was carried out in a single concentration – the addition of carbon and energy for the functional biomass was similar in every HQ dose – the changes observed when the supplemented HQ dose was increased may be explained by changes in HQ speciation, which might modify HQ ability to

intervene in electron transfer processes such as denitrification, CO<sub>2</sub> production and dehydrogenase activity in soil.

## Ethical disclosures

**Protection of human and animal subjects.** The author declares that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The author declares that no patient data appear in this article.

**Right to privacy and informed consent.** The author declares that no patient data appear in this article.

## Conflict of interest

The author declares to have no conflicts of interest.

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